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Effect of metabolic stressors on survival and growth of *in vitro* cultured ovine preantral follicles and enclosed oocytes

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ABSTRACT

The present study was undertaken to study the effect of metabolic stressors like elevated levels of ammonia, urea, Non-esterified fatty acid (NEFA) and β-hydroxybutyric acid (BHB) on preantral follicle growth, survival, growth rates of oocytes enclosed in preantral follicles (PFs), maturation rates of oocytes recovered from cultured follicles, hormone production (estrogen and progesterone), reactive oxygen species (ROS) as well as superoxide dismutase (SOD) activity. Small pre-antral follicles (SPFs, 100 −250 μm) and large pre-antral follicles (LPFs, 250−450 μm) were isolated from slaughterhouse ovaries by a mechanical cum enzymatic method. SPFs and LPFs were cultured *in vitro* for 14 and 7 days respectively and examined for their growth, survival and growth rates of enclosed oocytes in PFs exposed with different concentration of ammonia (0, 100, 150, 200, 250, 300 and 400 μM), urea (0, 4, 4.5, 5, 5.5,6, 7 and 8 mM), NEFA [Basal NEFA (70 μM): stearic acid, SA (10 μM)+Palmitic acid, PA(20 μM)+oleic acid, OA(40 μM), b) Medium combo (140 μM): SA (20 μM)+ PA(40 μM)+ OA(80 μM), c) High combo (210 μM): SA (30 μM)+PA(60 μM)+OA(120 μM), d) Very high Combo (280 μM): SA(40 μM)+PA(80 μM)+ OA(160 μM)] and BHB (0, 0.5, 0.75, and 1 μM). Results indicated that ammonia, urea, NEFA and BHB caused inhibition of survival and growth of *in vitro* cultured ovine PFs and enclosed oocytes at the levels of 300 μM, 8 mM, high combo level of NEFA and 0.75 μM respectively. Our study may contribute to the identification of the mechanisms involved in decline of fertility due to metabolic and nutritional stress in ruminants.

1. Introduction

The metabolites of protein digestion (ammonia and urea) affected different components of reproductive system of the ruminants as it was reported that ammonia affected the oocyte quality before ovulation, while urea mainly interfered negatively after fertilization [1]. Similarly, energy deficit diet led to lipolysis which was characterized by elevated non-esterified fatty acid (NEFA) and β-hydroxybutyric acid (BHB) along with low glucose concentrations in serum [2]. These nutrient metabolites affected endocrine signalling and the quality of the oocyte and/or embryo [3]. Moreover, during the negative energy balance (NEB) accumulation of NEFA (derived from adipose tissue) in follicular fluid hampered the proliferation of the granulosa cells and thus jeopardized the oocyte development [4]. We earlier reported that the total NEFA, BHB, ammonia and urea above the physiological concentrations in serum and follicular fluids had been considered as nutritional and metabolic stressors [5,6]. We had also shown that elevated ammonia and NEFA concentrations in the final maturation phase of oocytes *in vitro* were unfavourable for the oocytes developmental competence and subsequent embryo development [7,8].

It was never examined if or to what extent folliculogenesis, follicle quality, and the maturational competence of the enclosed oocyte in the preantral follicles (PFs) were affected by continuous exposure to elevated concentrations of ammonia, urea, NEFA, and BHB during follicle growth *in vitro*. Hence, the aim of this present study was to investigate the effects of exposure to elevated ammonia, urea, NEFA, and BHB concentrations on the frequency of follicular growth [both small preantral follicle (SPFs) and large preantral follicles (LPFs)], survival, oocyte maturation, reactive oxygen species (ROS) production, superoxide dismutase (SOD) enzyme activity and estrogen/progesterone production during *in vitro* culture of PFs in ovine model.

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2. Material and method

Unless otherwise stated, culture media and chemicals were purchased from Sigma Chemicals (St Louis, MO, USA).

2.1. Ovaries

Ovaries from mature, healthy, non-pregnant sheep (*Ovis aries*, age: 2–2.5 years) were collected from a local abattoir during breeding seasons (March to April, June to July and from September to October). Ovaries were brought to the laboratory in warm (32–35 °C) normal saline (0.9% NaCl) containing 50 μg / mL gentamicin sulfate within 1 h of slaughter.

2.2. Recovery and culture of pre-antral follicles

A combined mechanical and enzymatic method developed in our laboratory was used to isolate PFs from sheep ovaries [9]. PFs with normal follicular outline, compact granulosa cells and visible oocyte were only selected. PFs were classified according to the criteria described earlier [9,10]. The diameter of SPFs, Fig 1 ranged from 100 to 250 μm and had 2-4 layers of granulosa cells. The diameter of LPFs, Fig. 2 ranged from 250 to 450 μm and had more than 4 layers of granulosa cells although they occasionally processed small antral cavity [10]. The isolated PFs (SPFs and LPFs) were washed in the isolation and washing medium containing minimum essential medium (MEM) supplemented with bovine serum albumin (BSA, 0.3%), glutamine (2 mM), sodium pyruvate (0.23 mM), hypoxanthine (2 mM) and gentamicin (50 μg/mL).

Only viable PFs as assessed by trypan blue staining method [11] were used for culture. In brief, PFs were incubated in 0.04% trypan blue for 2 min at room temperature. PFs stained with trypan blue were considered as dead (Fig. 3) and unstained PFs were considered as alive (Fig. 4). The isolated PFs (two to three in a group) were transferred in 100 μL droplets of the culture medium under paraffin oil in a 35-mm petri dish and cultured in a CO_2 incubator (38.5 °C, 5% CO_2 in air, 90–95% relative humidity) for 7 days (LPFs) or 14 days (SPFs). The control culture medium was MEM supplemented with BSA (0.3%), glutamine (2 mM), sodium pyruvate (0.23 mM), hypoxanthine (2 mM), insulin–selenium–transferin (1%) and gentamicin (50 μg/ml) and FSH-P (7 μg/ml; biological potency = 7 U/mg; F2293; LH ≤ 1%). The first day of culture was designated as Day-0. The medium was replenished at the end of Day-2 and Day-4 for 7 day culture and additionally at the end of Day-6, Day-8, Day-10 and Day-12 for a 14 day culture.

Follicle diameters (μm) were assessed by measuring the distance between 2 sides of the follicle, straight through the center of the oocyte using an eye piece micrometer fitted on the stero zoom microscope (magnification ×200) [12]. Oocyte diameters included zona-pellucida thickness as it was reported that the formation of the zona pellucida always occurred during the PF stage [13]. The final diameter of the follicles was recorded together with the presence and absence of an antral cavity (a visible translucent area within the granulosa cell mass (Fig. 5). The growth rate measured

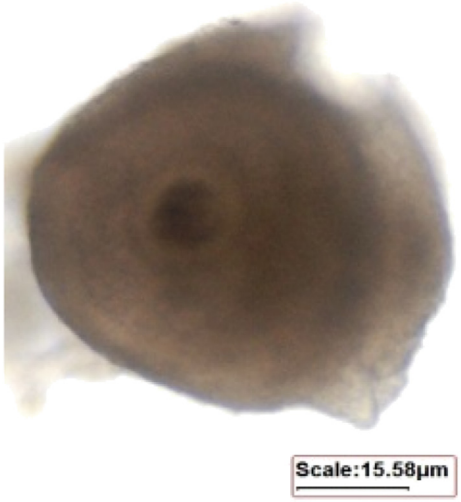

Fig. 1. Small Preantral follicle.

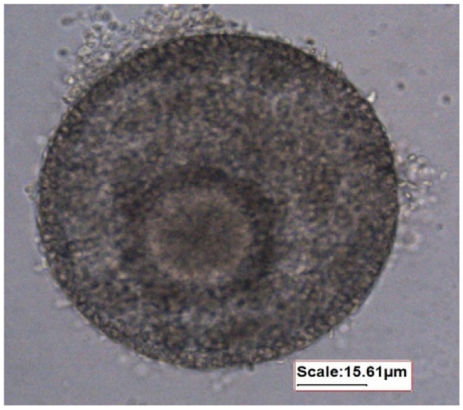

Fig. 2. Large preantral follicle.

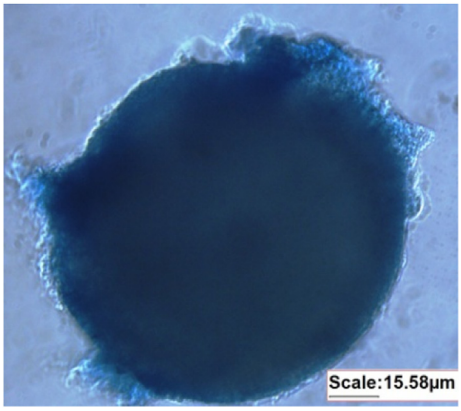

Fig. 3. Preantral follicles (Dead).

Fig. 4. Preantral follicle (alive).